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Colicin V Virulence Plasmids

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INTRODUCTION

ColV virulence plasmids are a heterogeneous group of large plasmids of the IncFI incompatibility group which encode the production of a colicin known as colicin V. Colicins are antibiotic substances that are produced by certain members of the family *Enterobacteriaceae* and that have lethal action against related sensitive strains (41). As the name implies, colicins were originally thought to be produced predominantly by *Escherichia coli* strains (43). Other bacteria were later found, however, that produce similarly acting substances, requiring the broader term "bacteriocins." Among the colicins so far described are colicins V, A, B, Ia, Ib, K, N, and E1 (84). Like F factors, colicinogenic factors were described before it was recognized that these factors were encoded by extrachromosomal DNA molecules, or plasmids (40). Unlike other colicin-encoding plasmids, ColV plasmids are found primarily among virulent enteric bacteria and have been shown to encode several virulence-related properties in addition to colicin V. How ColV plasmids contribute to the virulence of enteric bacteria is the subject of this review.

The colicin V phenomenon was first described 66 years ago (48), but strains carrying ColV plasmids are still being isolated from clinical specimens. ColV plasmids have thus survived the changing selective pressures of the past 66 years, an era including the advent and widespread use of antibiotics. Indeed, the use of antimicrobial agents may have selected for plasmid carriage of the virulence-related properties encoded by ColV plasmids, since these plasmids are in most cases transferable and could be spread from an antibiotic-sensitive strain to a resistant strain in vivo, providing "quantum leaps" toward virulence (34). The low copy number of ColV plasmids may also have played a role in favoring ColV plasmid carriage of virulence factors that cannot be tolerated in high gene dosages. Such issues are incompletely understood at present. Systematic studies of ColV plasmids are needed to address these and other questions, including the following. (i) Which virulence factors are encoded by which particular ColV plasmids? (ii) Are these virulence properties associated with particular clinical conditions? (iii) How do the various virulence properties contribute to the overall pathogenicity of the bacterial host? (iv) What controls the expression of these properties? (v) How important are plasmid copy number, plasmid replication, incompatibility group, and host range in the expression of these virulence properties? (vi) Could ColV plasmids be used as a model system to study the epidemiology, evolution, and molecular biology of other large virulence plasmids? Studies which have begun to address these questions are reviewed herein.

In the past it has often been assumed that observations derived from one ColV plasmid could be applied to all ColV plasmids. More recently it has been found that the ColV group is a diverse assortment of IncFI plasmids (114). All of these plasmids by definition encode colicin V, but may or may not encode other properties related to virulence. The contribution of ColV plasmids to virulence is a complex issue, owing to their diversity and size, since they range from 80 to 180 kb (Table 1), and also to the complexity of the individual factors. Properties that have been associated with ColV plasmids include colicin V, plasmid transfer-related functions, the aerobactin iron uptake system, increased serum survival, resistance to phagocytosis, change in motility, hydrophobicity, and intestinal epithelial cell adherence. (In addition to conjugal mobility of the entire plasmid is the potential for genetic mobility of some of the individual plasmid-encoded properties.) The ColV plasmid properties have distinctive but complementary roles during the infection process. ColV plasmids thus present a unique transferable package of virulence properties for the study of invading organisms which must pass through different environments of the vertebrate host.

HISTORICAL OVERVIEW

In 1925 Andre Gratia first described the colicin V phenomenon (48), calling it "principle V." It was named after a "colibacillus" strain which was virulent for animals and which Gratia called *coli* V. These cells "appeared normal" but were capable of causing the lysis of other cells, suspi-

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TABLE 1. Distinguishing characteristics of ColV plasmids

Plasmid	Size (kbp)	No. of copies of IS*1* element	Size (kbp) of *Hin*dIII fragment carrying: IS*1* sequence upstream from aerobactin genes	Size (kbp) of *Hin*dIII fragment carrying: Downstream IS*1* and aerobactin genes	Known phenotypes: Aerobactin system	Known phenotypes: Other(s)
pColV-P72	137	3	8.6	15.5	+	
pColV-B188	80	2	8.6	45	+	Adherence,[a] Tra^{-b}
pColV-292	150	4	8.6	14.5	+	Serum resistance[c]
pColV-H247	137	3	8.6	15.5	+	
pColV-CA7V	98	3	15.8[d]	NA[e]	−	Tra^{-}
pColV-K30	144	5	8.6	16.3	+	
pColV-F70	150	4	8.6	14.5	+	Serum resistance
pColV-F54	150	4	8.6	14.5	+	Serum resistance
pColV-K229	180	7	8.6	14.5	+	
pColV-K311	130	2	8.6	45	+	
pColV-K328	140	2	8.6	45	+	

[a] Plasmid-associated bacterial adherence to intestinal epithelial cells (21).
[b] Transfer deficient (54, 97).
[c] Plasmid-associated bacterial growth in rabbit serum (see the text and reference 79).
[d] In pColV-CA7V, all three copies of IS*1* are contained within this 15.8-kbp *Hin*dIII fragment, but the copy which is linked to REPI sequences is partially deleted.
[e] NA, Not applicalbe since pColV-CA7V is genotypically and phenotypically negative for the aerobactin iron uptake system.

ciously similar to the lysis caused by bacteriophages. Gratia began characterization of the substance responsible for this lysis of sensitive cells. He found that, unlike phages, this substance did not regenerate and that it did not produce localized plaques typical of phages (48). He was able to obtain resistant cells from a sensitive population, and he noticed that this "antibiotic" substance diffused readily (this property distinguished the *coli* V principle from similarly acting substances), it passed through membranes impermeable to bacterial cells, and it was stable in chloroform and serum (48). Later called colicin V (43), the activity appeared to be specific for certain *E. coli*, *Shigella*, and *Salmonella* strains (43, 96). Fredericq and Joiris surveyed a number of these strains and found that pathogenic cells produced colicin V at about twice the frequency of nonpathogenic cells (42). This observation raised the question of the potential role of colicin V in virulence.

It was demonstrated in 1962 by Nagel de Zwaig et al. that the colicin V phenotype was transferable (74). The concept of genetic transfer preceded the characterization of plasmids as extrachromosomal self-replicating genetic elements. In 1965 DeWitt and Helinski, using cesium chloride density gradient centrifugation, demonstrated that the similar factor ColE1 was an autonomous genetic element (31). This type of experiment had also been performed with the F factor by Falkow et al. (35). In 1967 it was shown that ColV factors were F-like in that were rapidly and efficiently transferred in exponentially growing cultures, and that the cells harboring these ColV factors were sensitive to "male-specific" RNA phages (65). In 1976 Smith and Huggins confirmed that most ColV plasmids were transferable; they also presented data from mixed-infection studies suggesting a role for colicin V in bacterial virulence (97). Smith had previously shown that 67% of *E. coli* strains causing bacteremic infections in poultry produced colicin V (96). However, in 1979, Quackenbush and Falkow used transposon mutagenesis to show that bacteria carrying a ColV plasmid with a mutated colicin V structural gene were as virulent to mice injected intraperitoneally as the nonmutant ColV plasmid-carrying bacteria (89). The implications of these findings will be discussed below.

Another work appearing in 1979 shifted the focus away from the study of colicin V as the primary virulence factor of ColV plasmids. Williams showed that the enhanced virulence associated with a ColV plasmid could be neutralized in vivo by coinjecting iron with the bacterial inoculum into animals, and that the enhanced in vitro growth rate obtained with ColV-carrying cells over plasmidless cells in media containing transferrin could be abolished if iron was also added to the media (117). These two observations indicated that the ColV plasmid was responsible for an iron uptake system. Spectrophotometric data indicated that the iron chelator was distinct from enterochelin, commonly produced by strains of *E. coli* and other enteric bacteria (117). The ColV iron uptake system was found to encode an hydroxamate iron-chelating siderophore identified as aerobactin (12, 113), the siderophore first discovered in culture supernatants of "*Aerobacter aerogenes*" 62-1 (46). At this time, the concept of a virulence property which facilitated bacterial growth under conditions of iron stress (117), such as in serum, was a novel concept. The concomitant discovery of an analogous plasmid-mediated iron uptake system in highly virulent strains of the fish pathogen *Vibrio anguillarum* (23) gave credibility to this concept, since in both plasmid-mediated systems the loss of the relevant plasmid greatly reduced virulence (23, 91, 97).

The plasmid pColV,I-K94, which does not specify an iron uptake system but nonetheless affects the 50% lethal dose (LD_{50}) of the bacterial host, was shown to encode a property correlated with enhanced bacterial survival in fresh rabbit serum and called *iss* (for increased serum survival) (10). In 1981 another virulence-related phenotype was attributed to a ColV plasmid: increased in vitro adherence by ColV plasmid-containing *E. coli* cells to mouse intestinal epithelium (21). Eventually, eight phenotypes were attributed to ColV plasmids. Since 1981, most investigators of ColV plasmids have focused their attention on the aerobactin iron uptake system. The distribution of the aerobactin system in *E. coli* isolates from humans and the virulence of avian *E. coli* strains as a function of aerobactin production were investigated (16, 62). In these studies, the presence of the aerobactin system was often presumed to indicate the presence of the ColV plasmid. The risk of this practice became clear when the aerobactin system was found to be chromosomally encoded in *E. coli* K1 (108), enteroinvasive *E. coli* (66), and

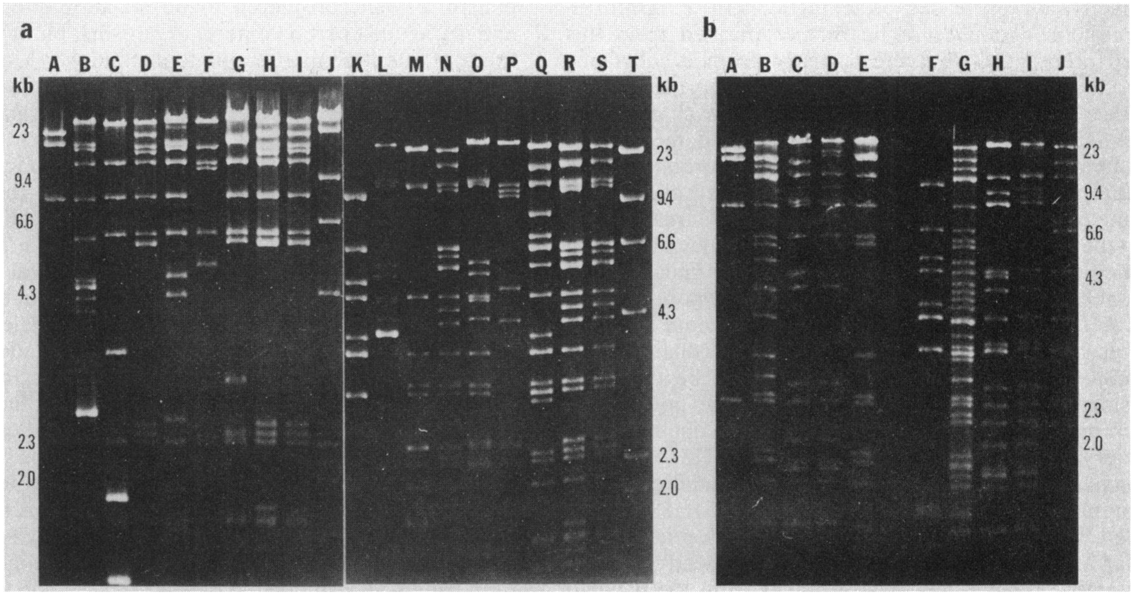

FIG. 1. Electrophoretic profiles of ColV plasmid DNA digested with restriction enzymes *Hin*dIII and *Hin*dIII-*Bam*HI. (a) Lanes: A and K, pJHCP1, a cloned derivative of pColV-K30 (85); B and L, pColV-P72; C and M, pColV-B188; D and N, pColV-292; E and O, pColV-H247; F and P, pColV-CA7V; G and Q, pColV-K30; H and R, pColV-F70; I and S, pColV-F54; J, pJHCP1 restriction enzyme digested with *Xho*I and lambda DNA digested with *Hin*dIII as molecular weight standards; T, lambda DNA digested with *Hin*dIII. Lanes A through I are *Hin*dIII-digested samples, and lanes K through S are *Hin*dIII-*Bam*HI-digested samples. (b) Lanes: A and F, pJHCP1; B and G, pColV-K229; C and H, pColV-K311; D and I, pColV-K328; E and J, pColV3-K30. Lanes A and E are *Hin*dIII-digested samples, and lanes F through J are *Hin*dIII-*Bam*HI-digested samples.

Shigella flexneri (63). In 1983, the genes for the enzymes that make up the biosynthetic pathway for the siderophore aerobactin, as well the structural gene for the outer membrane receptor for ferric aerobactin, were cloned from the plasmid pColV-K30 (8). In subsequent years several groups characterized the aerobactin systems operon (9, 13, 17, 27, 29, 52) and the flanking genetic regions (66, 85–87, 114) in terms of genetic structure, function, and regulation.

Questions related to the plasmid biology of the ColV plasmids have only recently been addressed. Using pColV-K30, investigators in our laboratory have begun to characterize the primary replicon of ColV plasmids, REPI, as well as the genetic loci responsible for plasmid incompatibility and partitioning (45, 85–87). As members of the IncFI incompatibility group, ColV plasmids were considered entirely F-like in their replication and incompatibility properties (59, 60, 65), but important differences have been found (45). The genetic linkage of the aerobactin system and universally conserved replication region REPI found in a collection of ColV plasmids (114) raised the issue of the suitability of these IncFI plasmids in carrying the aerobactin system, especially since this "ColV-aerobactin genetic system" (115) (see below) has never been found on a non-IncFI plasmid. Postulated mechanisms for genetic mobility of the aerobactin system should incorporate this genetic linkage. The suitability of the IncFI ColV plasmids in relation to the plasmid carriage of the other properties has not been explored, although colicin V also appears to be carried only by such IncFI plasmids.

PROPOSED VIRULENCE-RELATED PHENOTYPES

It should be emphasized that the virulence-related phenotypes ascribed to ColV plasmids are not held in common by all of the ColV plasmids studied. Except for the aerobactin iron uptake system (114), the distribution of the other phenotypes among prototypic ColV plasmids is largely unknown. Thus far, it appears that most ColV plasmids carry, in addition to colicin V, the virulence determinant aerobactin, and some are known to carry other virulence determinants such as for serum resistance (Table 1). In compiling these data from clinical isolates, investigators have faced problems in identifying ColV plasmids. Only in recent years has there been some convention in nomenclature; without molecular fingerprints in the form of restriction enzyme digests (Fig. 1), some plasmids received more than one designation or the designation of the host strain, and some distinct plasmids were given the same designation.

Colicin V

Most characterized colicins range in molecular mass from 27,000 to 80,000 Da and are inducible by conditions which activate the SOS DNA repair response (54, 61). Colicin V, on the other hand, is a small molecule and is not SOS inducible (56); it is therefore unusual among colicins. Furthermore, colicin V is not released from the bacterial cell by means of cell lysis (the typical mechanism for most colicins), but rather appears to be exported (47). Because of these properties, colicin V could be classified in the newly described group of lower-molecular-weight bacteriocins called microcins (3). However, there are historical reasons to keep the original classification. Colicin V was the first colicin to be described, in 1925 by Gratia. At that time Gratia reported that colicin V was stable for 30 min at 120°C (48). Years later colicin V was believed to be an unstable protein, but an effective method of stabilization was developed by using steam treatment of culture supernatants (122), which apparently inactivated proteases attacking colicin V. Unfortunately, colicin V could not be further purified from this stabilized preparation, but the partially purified substance was used to show an inhibition of active transport across the membranes of colicin V-sensitive cells and a lack of inhibition in colicin V-resistant cells (122). This was demonstrated by a failure of the cells to take up radiolabeled proline. It was found that colicin V-treated cells could not generate a membrane potential, in contrast to the colicin V-treated cells carrying the colicin V immunity genes. It is believed that the effect of colicin V is not to induce increased permeability by ion channel formation, the effect seen with the pore-forming colicins A, B, EI, Ia, Ib, and K (84), since solute accumulation was not observed in colicin V-treated cells. Rather, the specific target of colicin V is thought to be the inner cytoplasmic membrane, where the membrane potential is disrupted by an undefined mechanism (122). Colicin V does not lyse erythrocytes in solid or liquid media (18).

By maxicell analysis of *E. coli* carrying cloned colicin V and immunity genes from the plasmid pColV-B188, it was concluded that the colicin and immunity peptides have molecular masses of 4,000 and 6,500 Da, respectively (44). Subsequently, minicell analysis of clones from the plasmid pColV-K30 showed the colicin V and immunity peptides migrating at 6,000 and 7,000 Da, respectively (47). In both studies, Tn*5* transposon mutants of the relevant clones were obtained and the genes were localized to approximately the same genetic region, 900 bp upstream from a *Bgl*II site. DNA sequencing of this 900-bp region should resolve the molecular mass disparities. The mechanism of colicin V immunity is unknown, although it is known that the immunity is specific for colicin V and does not protect against other colicins such as colicin Ia (122). (In general, colicin immunity involves a protein, encoded by the colicin-producing plasmid, which interacts with the colicin to prevent its action.)

In addition to the genes for the colicin V structural and immunity proteins, two plasmid genes are required for the export of colicin V. Thus, a total of four plasmid genes are required for colicin V synthesis, export, and immunity. An *E. coli* chromosomal locus, *cvpA*, is also required for colicin V production (36). The plasmid genes have recently been defined within a 4.2-kb region of DNA cloned from pColV-K30 by Glison et al. (47). They designated the immunity determinant *cvi*, the genes required for colicin V export *cvaA* and *cvaB*, and the structural gene for colicin V *cvaC*. By minicell analysis of the Tn*5* derivatives, the export proteins were estimated to be 43,000 and 27,000 Da, and it was suggested that their encoding genes are overlapping (47). In studies with iron chelators and *fur* regulatory mutants, it was found that colicin V synthesis is induced under conditions of iron limitation. Mutants with mutations in the *cir*, *tonB*, and *exbB* genes are resistant to colicin V, suggesting that these gene products are involved in colicin V uptake (18). The *cir* gene product is the outer membrane receptor for colicins Ia and Ib; it has also been suspected to be the receptor for colicin V, but direct evidence is lacking. Cir is involved in the transport of iron-chelating catechols and analog compounds (78), and its production is regulated by cellular iron content as well as by temperature (50, 51). Other colicins use receptor pathways involving either *tonB* or the *tolQRAB* gene cluster and therefore seem to parallel bacteriophage entry pathways (84).

In considering colicin V, it is important to make the distinction between its possible role as a toxin against eukaryotic cells and its possible role in enhancing the survival of the bacteria producing it. From its early history, colicin V was considered to be an antibiotic, with lethal activity against certain enteric organisms, but it was also the focus of attention in attempts to explain why ColV plasmids were carried by virulent invasive *E. coli* strains. Indeed, there has been a renewed interest in colicins, in some cases because they mimic activities of other toxic entities such as bacteriophage, diphtheria toxin, and antibiotics (84). Two studies were reported in 1979 that examined the possibility that colicin V is a toxin affecting eukaryotic cells. Quackenbush and Falkow obtained mutants with Tn*1* transposon insertion mutations in the colicin V structural gene of the ColV plasmid pColV-B188 (89). These mutants, when injected intraperitoneally, killed mice as efficiently as the parent ColV plasmid-containing strain. The authors speculated that some other genetic determinant for virulence enhancement could be closely linked to the colicin V-related genes. Soon thereafter, Binns et al. presented evidence that this was in fact the case in pColV,I-K94 (10). Using clones and deletion derivatives of this plasmid, they tested pathogenicity by intramuscular injection of chickens to obtain the LD_{50}. Loss of about 20 kb of this plasmid increased the LD_{50} from 10^5 to 10^7, regardless of whether the colicin V structural gene was also deleted. The critical region linked to the colicin V genes was found to encode a property which increased the survival of *E. coli* in chicken serum (10).

Neither of the above studies addressed the role of colicin V in enhancing bacterial survival in a mixed population. In such a physiological niche as the mammalian intestine, bacterial survival must involve successful competition with diverse bacteria: a successful pathogen must first be a successful parasite. Early studies by Smith and Huggins demonstrated the enhanced survival of colicin V-producing *E. coli* strains in the colonization of human volunteers (99). A mixture of *E. coli* organisms was consumed orally on the first day. Of these organisms, 10% produced colicin V by virtue of a nontransferable plasmid and 90% did not produce colicin V. Of the two cell populations, only the colicin V-producing *E. coli* strains were recovered by day 7. This experiment suggests that colicin V enhances intestinal survival of the host bacteria and thereby acts at the level of colonization. However, the role of colicin V remains in doubt for lack of the critical experiments: determining the survival and colonization of $ColV^+$ wild-type and $ColV^-$ derivative strains by using specifically mutated ColV genes in mixed infections via oral inoculation. The potential competitive advantage conferred by colicin V could not be assessed in experiments involving intraperitoneal injection of pure cultures; also, the specific role of colicin V in colonization and invasion following oral administration, the normal route of *E. coli* acquisition, has not been evaluated.

The recent finding that the colicin V genotype was predominantly chromosomal in diarrheal isolates and predominantly plasmid encoded in bacteremic isolates is circumstantial evidence for the greater importance of colicin V in the gut (37). For a bacterium to persist in the gut, possession of colicin V appears advantageous; for the bacterium to invade beyond the gut, additional phenotypic traits appear to be required.

Aerobactin Iron Uptake System

The demonstration that the genotype for the aerobactin iron uptake system was carried on the ColV plasmid linked two points: (i) the free iron concentration in the vertebrate host, at less than 10^{-12} M, is too low to sustain bacterial nutritional needs, which are approximately 10^{-7} M (67, 76,

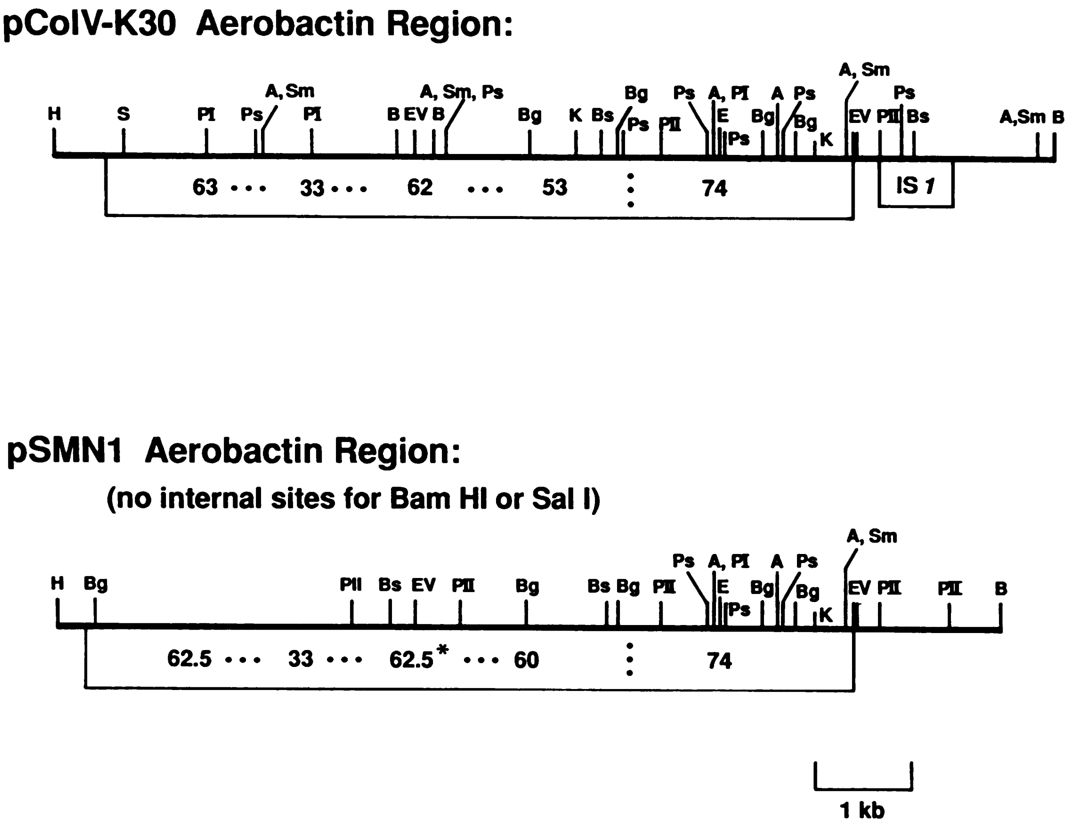

FIG. 2. Restriction enzyme maps derived from aerobactin system clones of *E. coli* 1315 pColV-K30 and *K. pneumoniae* ("*A. aerogenes*") 62-1 pSMN1. The numbers beneath the maps indicate the sizes of the encoded proteins in kilodaltons. The asterisks refers to the putative synthetase subunit molecular weight (115). Enzyme abbreviations: H, *Hin*dIII; S, *Sal*I; PI, *Pvu*I; PvII, *Pvu*II; A, *Ava*I; Ps, *Pst*I; Sm, *Sma*I; B, *Bam*HI; EV, *Eco*RV; Bg, *Bgl*II; K, *Kpn*I; Bs, *Bst*EII; and E, *Eco*RI.

77); and (ii) the correlation between ColV plasmid carriage and invasiveness of *E. coli* could be explained on the basis of a factor which provides the organism with the ability to cope with this restrictive free-iron concentration (49, 119). The aerobactin iron uptake system was initially explored by using three ColV plasmids: pColV-K30 (9), pColV-K311 (13), and pRJ100 (102). In 1979, Williams designed an experiment which added the dimension of iron to the mixed infection-type studies described above. Strains with and without pColV-K30 were used to inoculate pairs of mice intraperitoneally. One animal of each pair also received iron (117). Without the coinjection of iron, the results were similar to those of Smith and Huggins (99) for oral inoculation of humans: the colicin V-producing strain was recovered at a much higher proportion than that in the administered dose. However, with added iron, there was no enhanced recovery of colicin V-producing *E. coli*. Williams also presented data to show that colicin V-producing cells grew normally despite addition of the serum iron-binding protein transferrin to media, whereas cells lacking the ColV plasmid had a significantly decreased growth rate, as measured by the mean generation time (117). Although most *E. coli* strains produce the iron chelator enterobactin, the aerobactin siderophore has been found to be better suited to compete with serum transferrin for nutritional iron (24, 30, 38). Unlike enterobactin, aerobactin is recycled after the iron is released into the cell (14, 118), and, interestingly, it efficiently removes iron from transferrin but not from lactoferrin (68).

We surveyed a number of prototypic ColV plasmids and found that most do encode the aerobactin iron uptake system (Table 1). Aerobactin, a hydroxamate siderophore of 565 Da, is the product of a biosynthetic operon encoding enzymes for a four-step pathway (76). On the basis of field desorption mass spectroscopy, the siderophore aerobactin produced by ColV plasmid-carrying *E. coli* strains was found to be identical to the molecule produced by an "*Aerobacter aerogenes*" plasmid (115), the organism from which aerobactin was discovered (46). The "*A. aerogenes*" plasmid pSMN1 is neither a ColV nor an IncFI plasmid (116a). Also, when we compared the DNA sequences encoding the aerobactin system from the two plasmids, we found that the siderophore-specific genes and enzymes differed significantly, although the gene and protein for the ferric aerobactin receptor were conserved (Fig. 2) (115). The "*A. aerogenes*" 62-1 strain has recently been reclassified as *Klebsiella pneumoniae* 62-1 (ATCC 25306). On the basis of restriction enzyme site maps and Southern hybridization experiments, it appears that this second aerobactin genotype is the one carried by virulent K1 and K2 strains of *K. pneumoniae* (75, 115). A third, genetically distinct aerobactin system genotype has also been identified in our laboratory, in a clinical *Enterobacter cloacae* strain (25). The occurrence of these different aerobactin genotypes and enzymes appears to be due to the diversity of bacterial species, perhaps as a result of constraints imposed by the different inner membrane environments where initial steps of aerobactin biosynthesis take place (111). Among the *Salmonella*, *Shigella*, and *E. coli* strains, the observed aerobactin genotype has always been the "ColV genotype" (63, 66, 108, 114). In a recent survey with pColV-K30 radiolabeled probes, the observed homology to the aerobactin receptor probe in *E. coli* strains lacking homology to the siderophore-specific genes may actually have been the result of homology to the 75 bp of IS*1* DNA flanking the receptor gene included in the particular receptor probe that was used (37).

Since the discovery of the genes for the aerobactin iron uptake system on the chromosomes of *E. coli* (66, 108) and *Shigella flexneri* (63), estimates of the prevalence of colicin V, aerobactin production, and other properties have been made more carefully with regard to genetic location. Plasmid carriage of the aerobactin system has more often been found in veterinary isolates, whereas human isolates more often contain the chromosomally encoded aerobactin system genes (16, 107a, 110). Similarly, it has been noticed that alpha-hemolysin-producing *E. coli* strains isolated from animals more often have plasmid-encoded genotypes, whereas the isolates from human infections often have chromosomally encoded hemolysin genotypes (73).

Two studies have been done to analyze these phenomena. Valvano et al. found that 75% of the *E. coli* K1 human neonate isolates producing aerobactin carried the genes on the bacterial chromosome (110). This contrasted with the earlier studies of septicemic *E. coli* infections of livestock, in which 67% of the poultry isolates were carriers of ColV plasmids (96) (ColV plasmids commonly isolated from poultry were later found to encode aerobactin [114]). With the *E. coli* K1 strains, there was an association between the production of aerobactin or hemolysin and the outer membrane pattern of the strain, such that the membrane pattern clonal group could be used to predict the probability of aerobactin or hemolysin production. In a study of bacteremic human urinary tract infections caused by *E. coli*, 27% of aerobactin-producing human strains had plasmid-mediated aerobactin whereas 73% had chromosomally encoded aerobactin (58), similar to the aforementioned 75% of the *E. coli* K1 isolates. The authors of this study speculated that plasmid association of the aerobactin system was coselected with antibiotic pressure, since the strains with plasmid-mediated aerobactin production were often antibiotic resistant and isolated from compromised patients. It was not determined, however, whether the plasmids that carried the aerobactin system genes were the same plasmids that carried the genes conferring antibiotic resistance, nor was the incidence of colicin V production surveyed. Valvano et al. (110) found that colicin V production paralleled the plasmid-encoded incidence of aerobactin production in the *E. coli* K1 isolates. These data thus document that ColV plasmid carriage of virulence factors such as the aerobactin system is clinically significant, in this case in association with neonatal bacterial meningitis, even though isolates with chromosomal aerobactin system genes were more prevalent. Factors determining the plasmid or chromosomal localization of virulence genes remain obscure, but a higher incidence of plasmid carriage among animal strains and antibiotic-resistant human strains, throughout the world and especially in endemic areas, appears to be an emerging pattern. The higher incidence of plasmid carriage of virulence factors in domestic-animal strains is thought to be related to the liberal use of antibiotics in animal feed and in the treatment of veterinary disease. Other factors may include species-specific activities of certain plasmid virulence determinants such as *iss* (see below).

The study of the aerobactin system was stimulated by the discovery of this system on the plasmid pColV-K30 (113). The aerobactin iron uptake system genes were originally cloned in the laboratory of J. Neilands (8). In our laboratory, Perez-Casal and Crosa obtained a cosmid clone which contained large regions of DNA upstream and downstream from the aerobactin region (85). From this clone came two important observations: (i) inverted copies of the insertion sequence IS*1* flank the aerobactin system genes; and (ii) two distinct replication regions designated REPI and REPII surround the IS*1*-bound aerobactin system genes (Fig. 3). The former observation suggested that the aerobactin system genes could be genetically mobile by means of transposition or recombination via the IS*1* sequences. This might explain the ubiquity of the aerobactin system, found encoded on both plasmids and chromosomes among *E. coli*, *Shigella* and *Salmonella* species. The existence of the genetically linked replication regions likewise suggested a role for either or both regions in the preservation of the aerobactin system genes on ColV plasmids (see below).

Increased Serum Survival and Resistance to Phagocytosis

After establishing residence in the gut, pathogenic enteric bacteria must overcome numerous host defense mechanisms before they can invade extraintestinal spaces. Once an organism penetrates the intestinal mucosa and gains access to the blood, it confronts the bacteriostatic and bactericidal properties of serum. Immunoglobulins and nonspecific factors such as lysozyme, transferrin, and complement can lead to phagocytosis and/or bacterial killing. Resistance to serum killing is multifactorial and complex and has been attributed to various bacterial cell surface components, such as lipopolysaccharides, capsules, and surface proteins, most of which are chromosomally encoded. Plasmid-mediated factors have been identified which increase serum resistance, but their characterization is complicated by host bacterial factors such as the K and O antigens and even by bacteriophage lysogeny (4, 19, 100).

ColV plasmids have been implicated in both increased serum survival and resistance to phagocytosis. Some suspected plasmid determinants are components of the plasmid DNA transfer apparatus, including F pili, the surface exclusion protein TraT, and perhaps others, since the transfer-specific genes span about 30 kb and encode many functions. In plasmid pColV,I-K94, the *iss* serum resistance locus is linked to the colicin V genes but unlinked to the plasmid transfer region, which could include *traT* (10, 19). This distinction of genetic loci has been confirmed (11, 88) and is important, especially in light of the recent finding that the cloned *iss* genes product also mediates surface exclusion (19). Nilius and Savage investigated the possible role of transfer-related functions such as F pili in serum resistance (79). They used pairs of ColV plasmid-carrying strains which were repressed or derepressed for transfer, assayed by sensitivity to F pili-specific bacteriophage. Plasmid-free and phage-resistant (repressed for transfer) *E. coli* K-12 strains carrying pColV-F54 were rapidly killed in 5% rabbit serum, whereas the derepressed strain grew. Similar results were obtained with pColV-F70 and constitutively derepressed pColV,I-K94, but no such growth resulted in the derepressed strain carrying pColV-H247. These results suggest that serum resistance is not a function of plasmid transfer

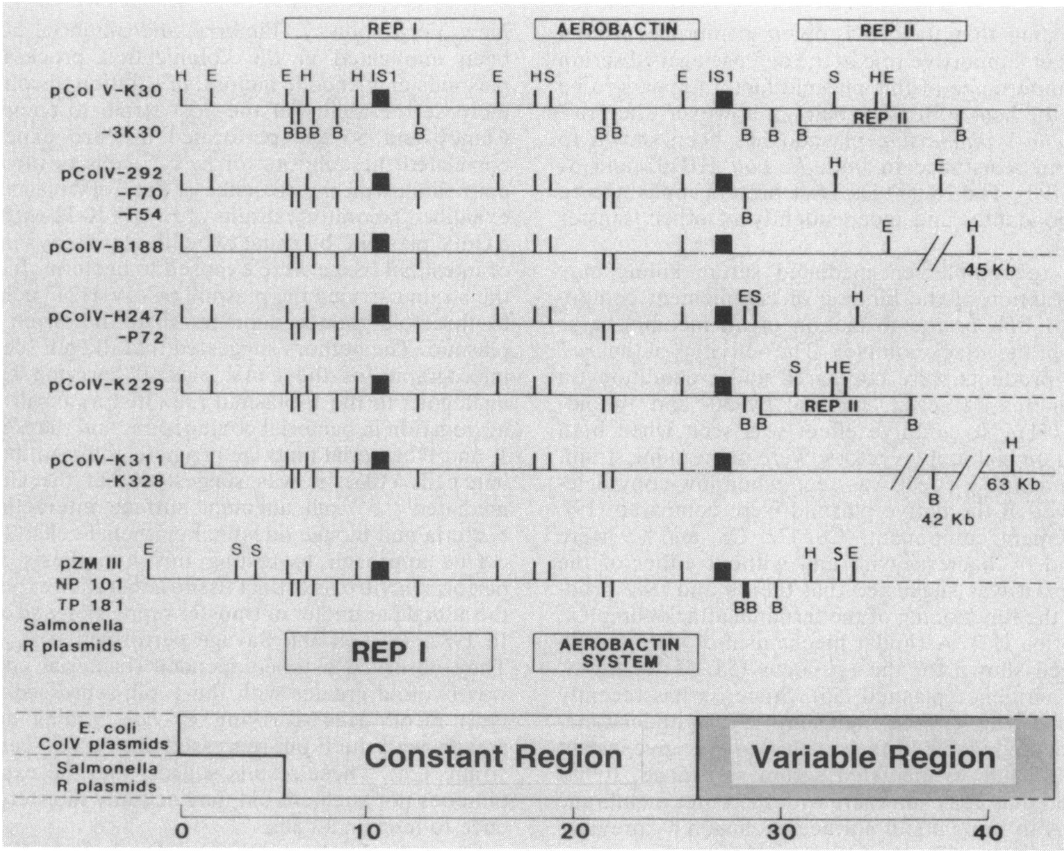

FIG. 3. Summary of the mapped regions of ColV plasmids (114) and of the *Salmonella* drug resistance plasmids pZM111, NTP101, and TP181 (22). The genetic regions held in common by all plasmids are described as the constant region, and the regions which vary among the plasmids are described as the variable region. The cleavage sites for restriction enzymes are abbreviated: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI, S, *Sal*I. The enzyme site location which coincide with those of pColV-K30 are indicated without letter designations.

status alone. It is of interest that the plasmids demonstrating increased serum resistance do so under conditions which derepress the cell for plasmid transfer, suggesting that environment signals, perhaps as found in serum, induce both bacterial conjugation and serum resistance.

The increased serum survival locus, *iss*, was identified by Binns et al. (10) and localized to a 5.3-kb *Hin*dIII-*Bam*HI fragment of plasmid pColV,I-K94. Because we did not include pColV,I-K94 in our molecular survey of ColV plasmids, and because of minor disparities in DNA molecular sizes as determined by electrophoresis in different laboratories and under different conditions, we suggest that this 5.3-kb *iss*-encoding fragment may be analogous to a 5.5-kb *Hin*dIII-*Bam*HI fragment found as a doublet only in ColV plasmids (of our survey) that engender serum resistance in host strains. Biological assays with specific clones and Southern blot hybridization experiments could test this postulate. In our molecular survey of ColV plasmids, we found *Hin*dIII-*Bam*HI fragments of approximately 5.0 kb conserved in all aerobactin-producing plasmids, doublet *Hin*dIII-*Bam*HI fragments of approximately 5.5 kb conserved in pColV-F54/70 and pColV-292 (Fig. 1a, lanes N, R, and S), and single 5.5-kb fragments in pColV-K30 and pColV-K229 (Fig. 1a, lane Q; Fig. 1b, lane G). In their analysis, Binns et al. found that deletion of the 5.3-kb fragment in recombinant derivatives resulted in a 100-fold increase in the LD_{50} of the host *E. coli* (10). Clones carrying this region enhanced bacterial survival and allowed growth in rabbit serum, whereas bacteria carrying clones without the *iss* region progressively lost viability. (Subsequently it was found that such increased survival in serum could be demonstrated with 50% rabbit and guinea pig serum, but only with dilute [<1%] human serum [11]). In a recent study of 346 human *E. coli* isolate, from diarrheal and bacteremic patients, it was found that 96% of the $ColV^+$ blood isolates contained DNA homologous to *iss* (37). If this population includes a variety of ColV plasmids, such a frequent occurrence of *iss*-homologous sequences would be unexpected, but could be due in part to cross-hybridization to the homologous membrane protein gene *bor* carried by strains which carry lysogenic lambda phage. Indeed, this locus has recently been shown to also result in increased survival in serum (4).

The TraT proteins are highly related (20, 57) membrane lipoproteins encoded by plasmids of incompatibility groups IncFI, IncFII, IncFIV, and probably others. TraT and TraS, encoded by genes within the transfer region of plasmid F, mediate surface exclusion (1). In plasmid conjugation, the bacterial cell becomes a poor recipient in matings with a donor cell carrying the same or related plasmid by virtue of surface exclusion. Cloned derivatives of *traT* also confer upon certain *E. coli* strains the ability to resist killing by fresh rabbit or guinea pig serum (70). In a survey of 680 clinical *E. coli* isolates, the *traT* gene was correlated with the presence of ColV plasmids, the K1 antigen, and R plasmids (71). However, only 58% of the serum-resistant isolates were *traT*⁺, suggesting that the TraT outer membrane protein plays a minor or supportive role in the defense against serum killing. The importance of this plasmid factor appears to be a function of the host strain (see below); however, the TraT of a *Salmonella typhimurium* plasmid has been shown to increase serum resistance in both *E. coli* HB101 and *S. typhimurium* (90). The 26,000-Da TraT protein appears to be expressed abundantly and independently of other transfer functions (57).

Resistance to complement-mediated serum killing may involve interruption of the binding of complement components prior to C5b or the formation of an incomplete or inactive membrane attack complex. The activities of the *traT* and *iss* gene products were compared under conditions in which they increased serum survival by 50- and 10-fold, respectively (11). No additive effect was seen when both genes, cloned on high-copy vectors, were in the same strain. However, an additive effect was seen when low-copy deletion derivatives of the native plasmid were compared (19). Since complement components C6, C7, C8, and C9 were equally bound by bacteria with and without either of the plasmid genes, it was suggested that the *iss* and *traT* products blocked the functioning of the terminal attack complex, not its formation (11). A similar mechanism of serum resistance has been shown for the *rck* locus (53, 55) of the *S. typhimurium* virulence plasmid (55). Since *iss* has recently been associated with surface exclusion (19), an understanding of the phenomenon of surface exclusion may give insight into both *traT*- and *iss*-mediated serum resistance. It has been proposed that TraT interacts with the outer membrane protein OmpA to bring about surface exclusion by preventing F pilus binding to OmpA, which would stabilize mating pairs (57). Alternatively, TraT could accomplish this destabilization by binding to the F pilus tip or by causing steric hindrance in a manner suggested for *Salmonella* O side chains (103a). However, studies with mutant TraT suggest that the phenomena of surface exclusion and serum resistance may be functionally separable (104).

Reduced phagocytosis of host *E. coli* by macrophages (83) has also been attributed to the TraT lipoprotein. Aguero et al. counted the numbers of encapsulated or unencapsulated *E. coli* cells ingested by macrophages and tested the effect of *traT* as cloned by using vectors of different copy number (2). The protective value of TraT was readily apparent in the unencapsulated strain, when cloned *traT* was used in either high- or low-copy vectors; in the encapsulated strain there was much less phagocytosis in all cases, and only with a high-copy *traT* clone was there a noticeable protective effect. With fluorescein-labeled anti-C3, an irregular pattern of C3 deposition was seen, so it was concluded that antagonism of opsonization was responsible for the effect on phagocytosis (2). These studies suggest that the host strain and copy number of the *traT* clone are critical in determining the impact of TraT on complement-mediated opsonization and killing and on subsequent phagocytosis. Although the native IncFI plasmids are low-copy plasmids, clinical strains often bear such inhibitors of phagocytosis as capsules. Thus, TraT-mediated serum resistance and antiphagocytic activity may be secondary in clinical invasive strains, but show dramatic effects in susceptible laboratory strains.

Intestinal Epithelial Cell Adherence, Hydrophobicity, and Motility

Competing with the resident flora to establish colonization in the intestinal tract has been likened to finding a seat on the New York subway. Fimbrial and afimbrial adhesins have been implicated in the colonization process, and ColV plasmids may encode factors, in addition to colicin V, which increase the ability of the host strain to compete. In 1981 Clancy and Savage performed in vitro experiments that correlated the adhesion of *E. coli* cells to mouse intestinal epithelium with the presence of the ColV plasmid (21). They examined laboratory strains of *E. coli* K-12 with and without a ColV plasmid, by using two different assays in which disks of intestinal tissue were exposed to bacteria. In both assays, the strain carrying the plasmid pColV-H247 adhered to two- to threefold-greater numbers than the strain without the plasmid. The authors suggested that the pili seen in electron micrographs of the ColV plasmid-carrying bacteria were analogous to the F-plasmid pili. Just as F pili promote cell aggregation in bacterial conjunction, and the K88, K99, type 1, and P bacterial fimbriae promote colonization of mammalian cells (105), it was suggested that the ColV plasmid-mediated "F" pili augment surface interactions between bacteria and mouse intestinal epithelial cells (21).

One approach to testing this hypothesis would be to perform in vitro intestinal tissue adherence experiments with the added parameter of transfer repression and derepression. In 1987, Darken and Savage performed such experiments. They observed a time-dependent bacterial adherence that was twofold greater with the F pili-repressed (phage-resistant) pColV-H247-carrying *E. coli* strains and threefold greater with the F pili-repressed pColV-F54-carrying *E. coli* strains (26). These results suggest that the expression of F pili does not augment but may actually interfere with adherence to intestinal cells.

Given the numbers of Fimbrial and afimbrial adhesins that have been implicated in the attachment of urinary and intestinal tract pathogens to the different cell types in the upper and lower urinary tract, adhesins other than the F pili could indeed mediate the colicin V-specific adherence effect. Some of the *E. coli* adhesins implicated in intestinal epithelial cell attachment include type 1 fimbriae (120); the enteropathogenic *E. coli* adherence factors EAF and LA (93); the enterotoxigenic *E. coli* fimbriae F18 (112), 987P (94), and F41 (72); and the K88, K99, and CFA colonizing-factor antigens (105). To date, plasmids encoding colicins B and I, but not colicin V, have also been found to encode the K88 antigen (39). Potential candidates for ColV-mediated adhesins would include the ColV-specified outer membrane proteins of molecular masses 119, 50 33, 28, and 26 kDa (TraT) and, less likely, the 74-kDa ferric aerobactin outer membrane receptor (7, 57, 80).

Since the observed colicin V-specific adherence effect is low level, it could be mediated by a nonspecific factor such as increased hydrophobicity. Tewari et al. correlated ColV plasmid carriage with increased hydrophobicity of the host *E. coli* strain as measured by a threefold increase in adherence to octyl-Sepharose (106). Shearing, which removes pili, and low temperature, which represses pili formation, reduced hydrophobicity, so it was concluded that conjugative pili were responsible for the effect. In another study, increased adherence to hydrophobic surfaces by enteropathogenic *E. coli* strains was correlated to the presence of mannose-resistant pili and to in vitro binding to rabbit intestine (32). The pilus-related increase in hydrophobicity has been attributed to divalent cations such as zinc and iron, which apparently bind to pili (103). Studies with conjugative pili mutants could resolve whether the ColV-mediated increase in adherence and in hydrophobicity is due to pili or to other factors. Such mutants could also be used to address

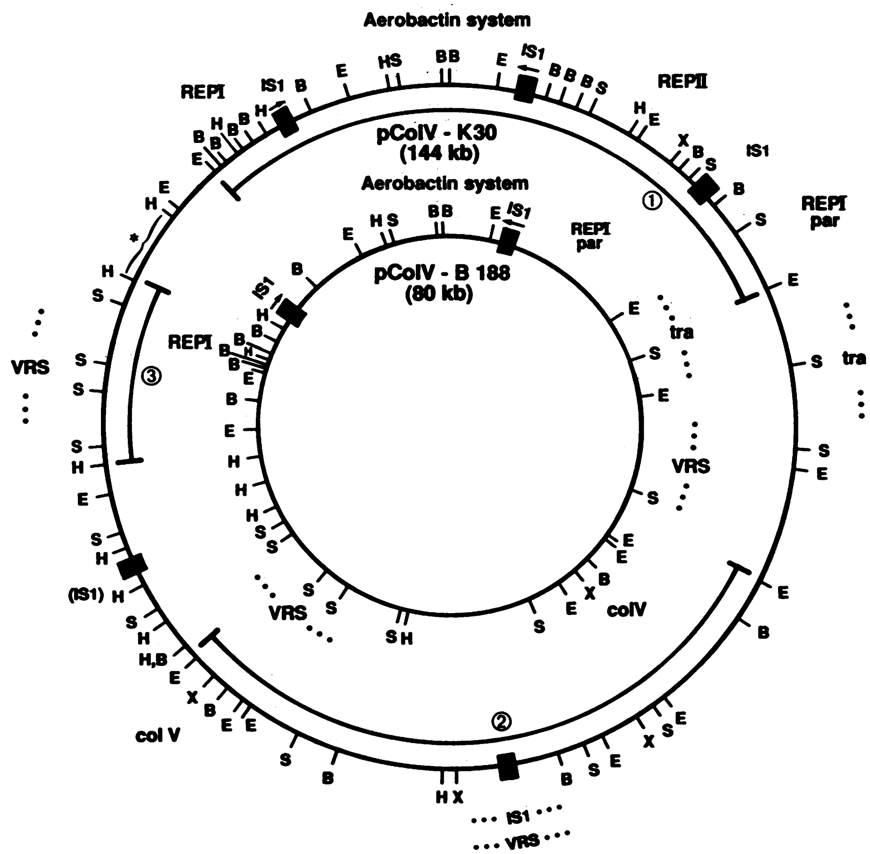

FIG. 4. Maps of pColV-B188 and pColV-K30, drawn to scale, such that the circumference of the pColV-K30 map is 1.8 times that of pColV-B188 (116). Indicated on the inside border of the pColV-K30 map are the boundaries of the clones used in mapping: 1, pJHCP1 (85); 2, pJHCP519; 3, pJHCVW26. The location of copies of the insertion sequence IS*1* is shown in cartoon fashion by black boxes which are not representative of the relatively small size of IS*1*. The arrows indicate the orientation of copies of IS*1* where known. The IS*1* copy which is believed to be partial, according to Southern blot hybridization, is labeled in parentheses. Dotted lines indicate that a particular phenotype location is approximate, within the limits of available restriction enzyme sites. The asterisks refers to a region containing five to seven small *Hin*dIII fragments in the bracketed area.

the role of these pili in ColV-mediated loss of motility, which was correlated to the autoagglutination (and perhaps to hydrophobicity) of ColV-bearing cells (107). The role of motility in the pathogenesis of invasive *E. coli* strains has not been determined; *S. typhimurium* mutants lacking flagella or motility have recently been found to be virulent in mice (64).

MOLECULAR CHARACTERIZATION OF COLV PLASMIDS

The most extensively studied ColV plasmids include pColV,I-K94, pColV-F54, pColV-H247, pColV-B188, and pColV-K30. The last four encode the aerobactin iron uptake system, as determined in a ColV plasmid genetic survey corroborated by bioassays (114). In this survey we confirmed the restriction enzyme pattern identity of pColV-K30 and pColV3-K30. (Also, pColV,I-K94 has been called pColV2, and pColV-CA7V has been called pColV1.) In extending this study of the aerobactin-related genes and flanking genetic regions, we have obtained the entire genetic maps of pColV-K30 and pColV-B188, based on restriction enzyme patterns and hybridization experiments with genetic probes for known phenotypic characteristics (Fig. 4) (116). With these maps in hand, other ColV plasmids should be more easily mapped, and the questions outlined in the introduction of this review may be more easily addressed molecularly.

Genetic Maps of pColV-B188 and pColV-K30

As the smallest known ColV plasmid, the 80-kb pColV-B188 plasmid (114) was the logical choice for the cloning and subsequent studies of colicin V. However, it was observed that the production of colicin V as encoded by pColV-B188 was reduced relative to that of other ColV plasmids (10). (The letter B in B188 means that the original *E. coli* strain carrying this plasmid was of bovine origin [98].) This *E. coli* strain was of serotype O78:H80, then the most common serotype among *E. coli* strains responsible for invasive infections of livestock (98), and it was used in the early virulence studies by Smith and Huggins because it was unusual in that the plasmid was not transferable (97). It is conceivable that the loss of transmissibility and, perhaps, a defect in colicin V expression are the result of a DNA deletion from an ancestral ColV plasmid (see below).

The plasmid pColV-K30 is the most intensively studied

ColV plasmid. It is a large plasmid, at 144 kb (85), but the focus of studies has been the 40 kb encompassing the aerobactin system genes and the flanking regions. The transfer region appears to encompass about 30 kb, as in the F plasmid, and the colicin V genes encompass about 4 kb, leaving 70 kb of DNA uncharted. In addition to the aerobactin iron uptake system and colicin V genes of pColV-K30, genetic probes have identified regions homologous to *traT* but not to *iss* (37).

From our genetic survey came the discovery of the following highly conserved genetically linked unit: the IncFI-correlated replication region REPI, the aerobactin iron uptake system flanked by inverted copies of the insertion sequence IS*1*, and a stability locus *par* which maintains REPI-driven clones (Fig. 4). This motif is also seen in the IncFII plasmid NR1, in which antibiotic resistance determinants are flanked by inverted copies of IS*1* and further flanked by the replication region *repA* and stability locus *stb* (121). For NR1, comparative analysis of the G+G percentages along the map illustrates the potential incorporation of distinct blocks of genetic regions coming together to build the resulting plasmid (121). Whether R determinant or aerobactin system, this genetic layout suggests how the nesting of the determinants between replication and stability functions might preserve a central determinant in surviving plasmid-carrying cells, since deletion events involving replication or partitioning functions would lead to plasmid loss from the cell population.

It is apparent from these studies that many restriction fragments are held in common among ColV plasmids. Many of these fragments are within the conserved replication-aerobactin system genetic unit, but some conserved fragments shared by all of the ColV plasmids investigated remain entirely cryptic. Some of these conserved fragments are also shared by IncFI non-ColV plasmids, such as the *Salmonella* R plasmids (Fig. 3) (22). Awaiting definitive identification is another of these highly conserved regions, tentatively designated VRS (for ColV repeated sequence) (Fig. 4). Good candidates for such identification are the insertion sequences IS*3* and IS*2*.

Although plasmid pColV-B188 is defective in transfer, it was found to contain DNA homologous to our *traF* probe. This probe was made from a self-replicating deletion derivative of F created by ligation of *Eco*RI fragments 3 and 5 (95). The homologous region was found to be within a large *Hin*dIII-*Bam*HI fragment in all ColV plasmids. Obvious size differences of the transfer regions were inapparent until detailed mapping suggested that possible deletion events could have resulted in the transfer-defective phenotype in pColV-B188 (Fig. 4). In our ColV plasmid survey, we performed Southern blot experiments with the colicin V clone pBQ41 as a probe (116). As expected, a highly conserved 4.2-kb *Eco*RI fragment hybridized to this probe. This fragment resides within the large (45-kb) *Hin*dIII fragment of pColV-B188 and within various and generally smaller *Hin*dIII fragments in the other ColV plasmids. These and other data were incorporated in the maps (Fig. 4).

The conservation of the replication region REPI among all the ColV plasmids investigated (114) suggests that this replication region is the primary replicon in ColV plasmids. A common heritage of the F and pColV-K30 plasmids is demonstrated by virtue of the corresponding homologies: REPI is homologous to the F plasmid *repF1B* (in *Eco*RI fragment 7), and REPII is homologous to the F plasmid *repF1A* (in *Eco*RI fragment 5) (6). Not all ColV plasmids carry the RepII replicon (Fig. 3) (22a). It has been reported that a third replicon of F, *repF1C*, showed partial homology with pColV-K30 (6), but the location of this partial homology has not been mapped in pColV-K30. No functional third replicon has been found in pColV-K30 (85).

Mobility of the Aerobactin System Genes

The discoveries of the inverted copies of the insertion sequence IS*1* flanking the aerobactin system genes, and the ubiquity of these genes among bacterial chromosomes and ColV plasmids, raised the issue of the genetic mobility of the aerobactin system and of whether such mobility would be via recombination, which is *recA* dependent, or transposition, which is *recA* independent. The existence of the flanking inverted copies of IS*1* has led some to refer to the aerobactin system as a transposon, but direct proof of transposability is necessary. De Lorenzo et al. observed *recA*-independent replicon fusion in cells containing a target plasmid and clones of the aerobactin system carrying one or two copies of IS*1* (28). The frequency of cointegrate formation was 10^{-5} to 10^{-7}. Duplication of IS*1* in the fused product, which would be expected if IS*1* had mediated the cointegrate formation (81), was not investigated. Occasional resolution of cointegrates occurred when aerobactin clones with two IS*1* copies were used, but these experiments involved passage of the cointegrate through a *recA*$^+$ bacterial host. These authors speculated that resolution proceeded by means of a *recA*-dependent event, similar to the *recA*-dependent cointegrate resolution seen with IS*1*-flanked heat-stable toxin (ST) (28, 101).

In the experiments with aerobactin system clones (28), the overall frequency of replicon fusion and resolution was 10^{-7}. Such an infrequent event is consistent with the conclusions from our studies comparing the maps of native ColV plasmids, taken from presumably *recA*$^+$ clinical strains. If transposition had occurred to spread the aerobactin system genotype among replicons, a variety of flanking sequences would be expected to occur beyond the IS*1* sequences. Instead, we observed a universal conservation of the upstream copy of IS*1* and of the upstream flanking region including replication region REPI (114). The downstream flanking sequences beyond the downstream IS*1* had diverged in all cases, whereas the downstream IS*1* either was conserved in location or very closely approximated its position as mapped in pColV-K30. A lack of conservation of flanking sequences such as IS*1* and REPI was found in the chromosomally encoded aerobactin system of *E. coli* K1 (109). This pattern of conservation and divergence argues against transposition of the aerobactin system and is consistent with mechanisms for mobility such as *recA*-dependent recombination or cointegration and resolution. However, infrequent transposition cannot be ruled out. It has been reported that transposition, inverse transposition, and cointegrate formation occur at a significantly higher frequency in *S. typhimurium* than in *E. coli* (15), and the IS*1*-bound aerobactin system genotype carried by the IncFI *Salmonella* R plasmids has been found in the inverted orientation relative to the replication region REPI (22). We never observed such an inverted orientation in the ColV plasmids native to *E. coli*, so the host organism may indeed be critical in regulating the frequency of these IS*1*-mediated events, perhaps as a function of the activity of IS*1* protein InsA or of the particular integrative host factor IHF (123). Also, transposon and transposon excision may be less frequent in certain subclones than in the native plasmid in natural settings in which conjugation occurs, since transposon excision has been observed to be more frequent in genomes which replicate via a single-stranded DNA intermediate, such as the single-stranded DNA phage or conjugating plasmids (33). "One-ended transposition," which is mediated by a single insertion sequence as seen in atypical Tn*21* and Tn*1721* (5) and is explained by neither the conservative nor the replicative-cointegrative transposition models, could be considered as another possible mechanism of spread of the aerobactin genes. However, the linkage of this aerobactin system in all the IncFI plasmids to the replication regions REPI and the fact that this "ColV genotype" has never been found on a non-IncFI plasmid suggest that there are characteristics of the IncFI replicon essential for optimal expression of the aerobactin system genotype when plasmid encoded. Among the ColV plasmids, recombination of large blocks of DNA sequences, including REPI, seems to be the mobility mechanism most easily reconciled with the observations to date.

CONCLUDING REMARKS

It has become widely recognized that bacterial virulence is the consequence of multiple genetic loci. Even in infectious diseases associated with a single dominant virulence factor, such as cholera toxin, the complexities of the host-parasite interaction require consideration of multiple regulatory and structural gene elements for an understanding of the pathogenic process (69). The ColV plasmids provide investigators with an array of virulence factors, encoded in a single self-replicating and transferable plasmid, which appear to enhance the pathogenicity of *E. coli* at successive stages of infection: colicin V provides a competitive advantage in colonization of the gut; changes in hydrophobicity and bacterial surface proteins facilitate attachment to appropriate host cell surfaces; *iss* and antiphagocytosis factors mediate resistance to host defenses; and the aerobactin system permits the acquisition of iron from serum transferrin, thereby enhancing growth. Furthermore, transmissibility of the plasmid permits dispersal of these virulence factors within a host-adapted bacterial population. Molecular linkage of some of these factors, such as the colicin V genes to the genes for increased serum survival (*iss*), and the nesting of the aerobactin system genes between replication and maintenance regions suggest that individual virulence genes have an increased adaptive advantage if the linked genes are simultaneously present and the gene products can act coordinately.

The importance of the aerobactin system to virulence has recently been directly demonstrated in a mouse peritonitis model (91) and has been correlated with such conditions as septicemia (96) and meningitis (110). The loss of the increased serum survival locus in a ColV plasmid resulted in a 100-fold increase in the bacterial LD_{50} for chickens (62). However, definitive experiments testing how, when, and where the other ColV virulence factors exert their effects remain to be done, and interpretations may be less straightforward. It is also unknown whether any of the factors potentiate the effects of other ColV properties in any model system. A low iron concentration induces the aerobactin system and colicin V genes, but the regulation of the other factors remains unexplored. The association of these ColV virulence factors with the IncFI replicon has been observed but not explained. Lastly, the functional differences and vertebrate host specificities of strains carrying particular ColV plasmids are not understood. Few of the questions raised in the introduction of this review have therefore been adequately addressed.

Studies with combinations of virulence factors cloned on low-copy vectors (such as a mini-ColV plasmid) could be used to investigate the role of ColV plasmids in the complex, multistage invasive infection process. This comparative approach could also be used to study other families of large low-copy plasmids, such as the *Salmonella* virulence plasmids. These plasmids range in size from 50 to 285 kb and could be subgrouped according to restriction enzyme fragments and phenotypic traits (82, 92). The IncFI aerobactin-producing *Salmonella* R plasmids discussed in this review (22) are distinct from these other *Salmonella* plasmids. It would be of interest to see whether any of the ColV property genotypes are identical or related to genotypes of *Salmonella* (or other) virulence plasmids. Finally, genetic mapping of additional ColV and other large plasmids would help define the genetic relatedness of ColV and other virulence plasmids and perhaps would begin to explain the current observation that ColV plasmids appear to be uniquely carried by the *E. coli* bacterial species.

The ColV-aerobactin (115) and the colicin V determinants have been found only among plasmids of the IncFI incompatibility group. Although the specific roles of ColV plasmid loci in virulence remain topics of speculation, most of these speculations are readily testable and the ColV plasmids provide a context to explore pathogenesis at a level that is more complex than the simple reductionist approach to studying clones that has typified these early years of molecular microbiology. Further experimentation in this field is likely to yield important new insights into the ColV plasmids and also into the unifying properties of large plasmids involved in bacterial pathogenesis.

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